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Applicant has changed its name from ProFound Pharma A/S to Maxygen ApS with effect from 01 September 2000. A transcript from the Danish Companies Register has been submitted to the Danish Patent Office as proof of the change of name. Consequently, Maxygen ApS is the same Applicant as ProFound Pharma A/S.

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NEW FACTOR VII or VIIa -LIKE MOLECULE

FIELD OF THE INVENTION

The present invention relates to new factor VII (FVII) or factor VIIa (FVIIa) polypeptides, to conjugates between the FVII or FVIIa polypeptide and a non-polypeptide moiety, to methods of preparing such polypeptides and conjugates and the use of such polypeptide or conjugates in therapy, in particular treatment of a variety of coagulation-related disorders.

BACKGROUND OF THE INVENTION

Blood coagulation is a process consisting of a complex interaction of various blood components, or factors, which eventually gives rise to a fibrin clot. Generally, the blood components which participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins which are converted into an active form by action of an activator. One of these coagulation factors is FVII.

Coagulation FVII is a vitamin K-dependent plasma protein which is synthesized in the liver and secreted into the blood as a single-chain (FVII) glycoprotein with a molecular weight of 53 kDa (Broze & Majerus, J. Biol. Chem 1980; 255:1242-1247). The FVII zymogen is converted into an activated form (FVIIa) by proteolytic cleavage at a single site, R152-I153, resulting in two chains linked by a single disulfide bridge. FVIIa in complex with tissue factor (FVIIa ~~complex) is able to convert both factor IX and factor X into their activated forms, followed by~~ reactions leading to rapid thrombin production and fibrin formation (Østerud & Rapaport, Proc Natl Acad Sci USA 1977; 74:5260-5264).

Residues numbers 6,7,14,16,19,20,25,26,29 and 35 shown in SEQ ID NO 2 are gamma-carboxyglutamic acids residues in the Gla domain which is important for the activity of FVII.

The gene coding for human FVII (hFVII) has been mapped to chromosome 13 at q34-qter 9 (de Grouchy et al., Hum Genet 1984; 66:230-233). It contains nine exons and spans 12.8 Kb (O'Hara et al., Proc Natl Acad Sci USA 1987; 84:5158-5162). The gene organisation and protein structure of FVII are similar to those of other vitamin K-dependent procoagulant proteins, with exons 1a and 1b encoding for signal sequence; exon 2 encodes the propeptide and Gla domain; exon 3 a short hydrophobic region; and exons 4 and 5, the epidermal growth factor-like domains; and exon 6 through 8, the serine protease catalytic domain (Yoshitake et al., Biochemistry 1985; 24: 3736-3750).

Experimental three-dimensional structures of hFVIIa have been reported by Pike et al., PNAS. U.S.A., 1999; 96:8925-30 and by Kemball-Cook et al., J.Struct.Biol, 1999; 127:213-223.

Experimental three-dimensional structures of hFVIIa in complex with soluble tissue factor using X-ray crystallographic methods have been reported by Banner et al., Nature, 1996; 380:41 and by Zhang et al., J.Mol.Biol, 1999; 285: 2089.

Experimental three-dimensional structures of smaller fragments of hFVII have been reported by Muranyi et al., Biochemistry, 1998; 37:10605 and by Kao et al., Biochemistry, 1999; 38:7097.

Relatively few protein-engineered variants of FVII have been reported (Dickinson & Ruf, J Bio Chem, 1997;272:19875-19879, Kemball-Cook et al., J Biol Chem, 1998; 273:8516-8521, Bharadwaj et al., J Biol Chem, 1996; 271:30685-30691, Ruf et al., Biochemistry, 1999; 38:1957-1966.

Expression of FVII in BHK or mammalian cells have been reported (WO92/15686, WO91/11514 and WO88/10295).

Commercial preparation of hFVIIa is sold under the name NovoSeven® and contain a recombinant hFVIIa (rFVIIa). NovoSeven® is indicated for the treatment of bleeding episodes in hemophilia A or B patients. NovoSeven® is the only rFVIIa for effective, reliable treatment of bleeding episodes available on the market.

An inactive form of FVII in which arginine 152 and/or isoleucine 153 was modified has been reported in WO91/1154. These amino acids are located at the activation site. Inactivation of

FVIIa by a serine proteinase inhibitor has also been described in WO96/12800 and inactivation by carbamylation of FVIIa at the α amino acid group I153 has been described by Petersen et al., Eur J Biochem, 1999;261:124-129. The inactivated form is capable to compete with wild-type FVII or FVIIa for binding to tissue factor and inhibiting clotting activity. The inactivated form of FVIIa is suggested to be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction and thrombotic stroke.

The short-half-life of circulating rFVIIa was reported in Summary basis for approval for NovoSeven, FDA reference number 96-0597. The half-life was reported to be only 2.3 hours. Relatively high doses and frequent administration is necessary to reach and sustain the desired therapeutic or prophylactic effect. As a consequence adequate dose regulation is difficult to obtain and the need of frequent intravenous administrations imposes restrictions on the patient's way of living.

Another problem in current rFVIIa is the relative instability of the molecule with respect to proteolytic degradation. Proteolytic degradation is a major obstacle for obtaining a preparation in solution as opposed to a lyophilised product. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and in the case of emergencies quicker action, which potentially can become life saving. Attempts to prevent proteolytic degradation by site directed mutagenesis and a description of major proteolytic sites have been disclosed in WO88/10295.

A molecule with a longer circulation half-life would decrease the number of administrations necessary. Given the association of current FVIIa product with frequent injections, and the potential for obtaining more optimal therapeutic FVIIa levels with concomitant enhanced therapeutic effect, there is clearly a need for improved FVII or FVIIa-like molecules.

One way to increase the circulation half-life of a protein is to ensure that renal clearance of the protein is reduced. This may be achieved by conjugating the protein to a chemical moiety, which is capable of conferring a reduced renal clearance to the protein. Furthermore, attachment of a chemical moiety to the protein or substitution of amino acids exposed to proteolysis may effectively block a proteolytic enzyme from physical contact with the protein, and thus prevent degradation. Polyethylene glycol (PEG) is one such chemical moiety that has been used in the preparation of therapeutic protein products.

WO98/32466 suggests that FVII among many other proteins, may be PEGylated, but does not contain any further information in this respect.

BRIEF DISCLOSURE OF THE INVENTION

This application discloses improved FVII and FVIIa molecules, in particular recombinant hFVII and hFVIIa molecules, providing one or more of the aforementioned desired benefits.

Accordingly, in a first aspect the invention relates to a conjugate comprising i) a FVII or FVIIa polypeptide which comprises an amino acid sequence that differs from the amino acid sequence of the wild-type hFVII or hVIIa in at least one introduced and/or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety.

In another aspect the invention relates to a conjugate comprising i) an active FVIIa polypeptide which comprises an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in at least one introduced and/or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety.

In yet another aspect the invention relates to a conjugate comprising i) an inactive FVII polypeptide which comprises an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in at least one introduced and/or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety.

Additionally, the invention relates to a conjugate comprising i) an FVIIa polypeptide attached to a serine proteinase inhibitor or carbamylated at the amino acid residue I153 and

further comprising an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in at least one introduced and/or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety.

The conjugate of the present invention has one or more improved properties as compared to commercially available rFVIIa, including increased functional *in vivo* half-life, an increased plasma half-life, and/or increased bioavailability and/or reduced sensitivity to proteolytic degradation.

Consequently, medical treatment with a conjugate of the invention offers a number of advantages over the currently available rFVIIa compound, including longer duration between injections.

In a further aspect the invention relates to generally novel FVII polypeptides, which polypeptides form part of a conjugate of the invention. The polypeptides of the invention are contemplated to be useful as such for therapeutic, diagnostic or other purposes, but find particular interest as intermediate products for the preparation of a conjugate of the invention.

In a still further aspect the invention relates to a substantially homogenous preparation of a conjugate of the invention.

In still further aspects the invention relates to means and methods for preparing a conjugate or a polypeptide of the invention, including nucleotide sequences and expression vectors encoding a polypeptide or a conjugate of the invention.

In still further aspects the invention relates to a pharmaceutical composition comprising a conjugate, polypeptide or preparation of the invention in a lyophilised or a stable liquid formulation, preferably a stable liquid formulation.

In a still further aspect the invention relates to a pharmaceutical composition comprising an active FVIIa conjugate, an active FVIIa polypeptide or preparation of the invention and methods of treating a mammal with such composition. In particular the active FVIIa polypeptide, conjugate or composition may be used to treat patients having diseases resulting in inadequate blood coagulation in response to damage to blood vessels. This group of patients include, but are not limited to, hemophiliacs, hemophiliacs with inhibitors to FVIII and FIX, patients with thrombocytopenia, patients with liver diseases, or otherwise healthy people with severe bleeding problems, e.g. due to trauma or major surgery.

In a final aspect the invention relates to a therapeutic composition comprising an inactive FVII or FVIIa conjugate, an inactive FVII or FVIIa polypeptide or preparation of the invention and methods of treating a mammal with such composition. In particular the inactive FVII or FVIIa polypeptide, conjugate or composition may be used for treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction, thrombotic stroke etc.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the context of the present application and invention the following definitions apply:

The term "conjugate" is intended to indicate a heterogeneous molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties such as polymer molecules, lipophilic compounds, carbohydrate moieties or organic derivatizing agents. The term covalent attachment means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. The term "non-conjugated polypeptide" may be used about the polypeptide part of the conjugate.

The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". The term is intended to cover carbohydrate

molecules, although, normally, the term is not intended to cover the type of carbohydrate molecule which is attached to the polypeptide by *in vivo* N- or O-glycosylation (as further described below), since such molecule is referred to herein as "a carbohydrate moiety". Except where the number of polymer molecule(s) is expressly indicated every reference to "a polymer", "a polymer molecule", "the polymer" or "the polymer molecule" contained in a single-chain trimeric polypeptide of the invention or otherwise used in the present invention shall be a reference to one or more polymer molecule(s).

The term "attachment group" is intended to indicate an amino acid residue group capable of coupling to a non-polypeptide moiety such as a polymer molecule or a carbohydrate moiety suitable for blocking a receptor-binding site in accordance with the invention. Useful attachment groups and their matching non-polypeptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-polypeptide moiety	Conjugation method/Activated PEG	Reference
$-NH_2$	N-terminal, Lys	Polymer, e.g. PEG	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-SH	Cys	Polymer, e.g. PEG, Carbohydrate moiety	PEG-vinylsulphone PEG-maleimide *In vitro* coupling	Shearwater Inc Delgado et al, critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249-304 (1992)
-OH	Ser, Thr, OH-, Lys	Carbohydrate moiety	*In vivo* O-linked glycosylation	
$-CONH_2$	Asn as part of an N-glycosylation site	Carbohydrate moiety Polymer, e.g. PEG	*In vivo N*-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Carbohydrate moiety	*In vitro* coupling	
$-CONH_2$	Gln	Carbohydrate moiety	*In vitro* coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759-65
Guanidino	Arg	Carbohydrate moiety	*In vitro* coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc. Boca Raton, FI
Imidazole ring	His	Carbohydrate moiety	*In vitro* coupling	As for guanidine

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting an N-glycosylation site (with the sequence NX'S/TX'', wherein X' and X'' may be different or identical and is any amino acid residue except proline, N is asparagine and S/T is either serine or threonine). Although the asparagine residue of the N-glycosylation site is the one to which the carbohydrate moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site is present. Accordingly, when the non-polypeptide moiety is a carbohydrate moiety and the conjugation is to be achieved by N-glycosylation, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the parent polypeptide is to be understood as amino acid residues constituting an N-glycosylation site is/are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

In the present application, amino acid names and atom names (e.g. CA, CB, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) which are based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names e.t.c.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine ~~(His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine~~ (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues. The terminology used for identifying amino acid positions/substitutions is illustrated as follows: G124 indicates position #124 occupied by a glycine residue in the amino acid sequence shown in SEQ ID NO 2. G124N indicates that the glycine residue of position 124 has been substituted for an arginine residue. The numbering of amino acid residues made herein is made relative to the amino acid sequence shown in SEQ ID NO 2.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in US 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

"Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

"Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term "introduce" is primarily intended to mean substitution of an existing amino acid residue, but may also mean insertion of an additional amino acid residue. The term "remove" is

primarily intended to mean substitution of the amino acid residue to be removed by another amino acid residue, but may also mean deletion (without substitution) of the amino acid residue to be removed.

The term "FVII" or "FVII polypeptide" refers to a FVII molecule provided in single chain form.

The term "FVIIa" or "FVIIa polypeptide" refers to a FVIIa molecule provided in its activated two chain form, where the peptide bond between R152 and I153 of the single chain form has been cleaved.

The terms "rFVII" and "rFVIIa" refer to recombinantly produced FVII and FVIIa molecules, respectively.

The terms "hFVII" and "hFVIIa" refer to wildtype human FVII and FVIIa, respectively.

The term "catalytic site " is used to mean the catalytic triad consisting of S344, A242 and H193 of the FVII polypeptide.

The term "active FVIIa" or "active FVIIa polypeptide" is used to mean a FVIIa polypeptide, which is able to bind to tissue factor and further activate plasma factor X or IX. Preferably the active FVIIa polypeptide has a clotting activity between 25-100 % as compared to hFVIIa complex. More preferably the FVIIa polypeptide has a clotting activity between 30–100% as compared to wild type FVIIa. Even more preferably the FVIIa polypeptide has a clotting between 50-100 % as compared to wild type FVIIa. The clotting activity may be determined by any method known in the art as further discussed in the Methods section hereinafter.

The term "inactive FVII", "inactive FVII polypeptide", "inactive FVIIa" or "inactive FVIIa polypeptide" is used to mean a FVII/FVIIa polypeptide with reduced clotting activity as compared to wild-type hFVIIa. The inactive FVII or FVIIa polypeptide or conjugate is capable of competing with the wild-type FVII or FVIIa complex for binding tissue factor, thereby inhibiting clotting activity. Preferably, the inactive FVII or FVIIa polypeptide has < 1 % clotting activity compared to wild-type hFVII or hFVIIa. More preferably the inactive FVII or FVIIa polypeptide has < 0.05% clotting activity compared to wild type hFVII or hFVIIa. Most preferably the inactive FVII or FVIIa polypeptide has <0.01 % clotting activity as compared to wild type hFVII or hFVIIa.

The serine proteinase inhibitor attached to the catalytic site could be an organophosphor compound, a sulfanylfluoride, apeptide halomethylketone such as a Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-Glu-Glu-Arg chlormethylketone, Dansyl-Phe-Phe-Arg chlormethylketone and Phe-Phe-Arg chlormethylketone or a azapeptode.

The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value. As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time at which 50% of the dispensed polypeptide or conjugate molecules is still present in the circulation/plasma/bloodstream. Determination of serum half-life is often more simple than determining the functional *in vivo* half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternatively terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The polypeptide or conjugate is cleared by the action of one or more of the kidney, reticuloendothelial systems (RES), spleen or liver, by FSH-receptor-mediated elimination, or by specific or unspecific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functionality to be retained is normally selected from proliferative or receptor binding activity. The functional *in vivo* half-life and the serum half-life may be determined by any suitable method known in the art as further discussed in the Methods section hereinafter.

The term "increased" as used about the functional *in vivo* half-life or plasma half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically significantly increased relative to that of a reference molecule, such as an un-conjugated rFVIIa (e.g. NovoSeven®) as determined under comparable conditions.

The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g. by glomerular filtration, tubular excretion or degradation in the tubular cells. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), symmetry, shape/rigidity, and charge. Sometimes, a molecular weight of about 67 kDa is considered to be a cut-off-value for renal clearance. A reduced renal clearance may be established by any suitable assay, e.g. an established *in vivo* assay. Typically, the renal clearance is determined by administering a labelled (e.g. radiolabelled or fluorescence labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. The reduced renal clearance is determined relative to the corresponding non-conjugated polypeptide or the non-conjugated corresponding wild-type polypeptide under comparable conditions.

The term "reduced sensitivity to proteolytic degradation" is primarily intended to mean a conjugate which has reduced sensitivity to proteolytic degradation in comparison to non-conjugated wild type FVIIa under comparable conditions. Preferably, the proteolytic degradation is reduced by at least 10%, such as at least 25% (e.g. by 10-25%), more preferably by at least 35%, such as at least 50%, (e.g. by 10-50%, such as 25-50%) even more preferably by at least ~~60%, such as by at least 75% or even at least 90%. Most preferably, the proteolytic degradation~~ is reduced by 100%. Thus, preferably the conjugate of the invention has a reduced proteolytic degradation as compared to wild-type FVIIa of 10-100%, preferably 25-100%, more preferably 50-100%, and most preferably by 75-100%.

The term "parent FVII " or "parent polypeptide" is intended to indicate the molecule to be modified in accordance with the present invention. A typical parent FVII is the hFVII or hFVIIa. A "variant" is a polypeptide, which differs in one or more amino acid residues from a parent polypeptide, normally in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues. Examples of parent hFVIIa include the rFVIIa (NovoSeven ®).

Conjugate of the invention

In one aspect the invention relates to various specific conjugates comprising i) an FVIIa polypeptide which comprises an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in at least one introduced or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety. The amino acid residues to be introduced and/or removed are described in further detail in the following sections.

Preferably, the FVII polypeptide is an active FVIIa polypeptide, in particular an active hFVIIa polypeptide.

In another aspect the invention relates to a conjugate comprising i) an inactive FVII or FVIIa polypeptide which comprises an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in at least one introduced or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and ii) a non-polypeptide moiety. The amino acid residues to be introduced and/or removed are described in further detail in the following sections.

For instance, the FVII or FVIIa polypeptide is rendered inactive by being attached to a serine proteinase inhibitor or carbamylated at the α amino acid group I153.

The conjugates of the invention is the result of a generally new strategy for developing improved FVII or FVIIa molecules. More specifically, by removing and/or introducing an amino acid residue comprising an attachment group for the non-polypeptide moiety it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g. to ensure an optimal distribution of non-polypeptide moieties on the surface of the FVII or FVIIa molecule

and to ensure that only the attachment groups intended to be conjugated is present in the molecule) and thereby obtain a new conjugate molecule, which has or has not FVII activity and in addition one or more improved properties as compared to FVII and FVIIa molecules available today.

In preferred embodiments of the present invention more than one amino acid residue of the FVII or FVIIa polypeptide is altered, e.g. the alteration embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice. In addition to the removal and/or introduction of amino acid residues the polypeptide i) may comprise a serine proteinase inhibitor to inhibit the catalytic site of the polypeptide, and/or other substitutions or glycosylations which are not related to introduction and/or removal of amino acid residues comprising an attachment group for the non-polypeptide moiety.

The amino acid residue comprising an attachment group for a non-polypeptide moiety, either it be removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide i) and the non-polypeptide moiety ii) is to be achieved. For instance, when the non-polypeptide moiety is a polymer molecule such as a polyethylene glycol or polyalkylene oxide derived molecule amino acid residues comprising an attachment group may be selected from the group consisting of lysine and cysteine. When conjugation to a lysine residue is to be achieved a suitable activated molecule is, e.g., mPEG-SPA from Shearwater Polymers or oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614). The first will be illustrated further in the following disclosure.

In order to avoid too much disruption of the structure and function of the FVII molecule the total number of amino acid residues to be altered in accordance with the present invention, e.g. as described in the subsequent sections herein, (as compared to the amino acid sequence shown in SEQ ID NO 2) will typically not exceed 15. The exact number of amino acid residues and the type of amino acid residues to be introduced depend, i.a., on the desired nature and degree of conjugation (e.g. the identity of the non-polypeptide moiety, how many non-polypeptide moieties it is desirable or possible to conjugate to the polypeptide, where they should be conjugated, etc.). The exact number of amino acid residues and the type of amino acid residues to be removed depend, i.a., on the desired nature and degree of conjugation (e.g. the identity of the non-polypeptide moiety, whether and where in the polypeptide conjugation should be avoided, etc.). Preferably, the polypeptide part of the conjugate of the invention or the polypeptide of the invention comprises an amino acid sequence, which differs in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO 2, such as in 1-8 or 2-8 amino acid residues, e.g. in 1-5 or 2-5 amino acid residue from the amino acid sequence shown in SEQ ID NO 2. Thus, normally the polypeptide part of the conjugate or the polypeptide of the invention comprises an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO 2 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues.

In a preferred embodiment one difference between the amino acid sequence of the polypeptide i) and the amino acid sequence shown in SEQ ID NO 2 is that at least one and preferably more, e.g. 1-15, amino acid residues comprising an attachment group for the non-polypeptide moiety ii) has been introduced, preferably by substitution, into the amino acid sequence. Thereby, the polypeptide part is altered in the content of the specific amino acid residues to which the non-polypeptide moiety of choice binds whereby a more efficient, specific and/or extensive conjugation is achieved. For instance, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased to an optimised level, the renal clearance of the conjugate is typically significantly reduced, due to the altered shape, size and/or charge of the molecule achieved by the conjugation. Furthermore, when the total number of amino acid residues comprising an attachment group for the non-polypeptide of choice is increased a greater proportion of the polypeptide molecule is shielded by the non-polypeptide moieties of choice leading to a lower immune response.

The term "one difference" as used in the present application is intended to allow for additional differences being present. Accordingly, in addition to the specified amino acid difference, other amino acid residues than those specified may be mutated.

In a further preferred embodiment one difference between the amino acid sequence of the polypeptide i) and the amino acid sequence shown in SEQ ID NO 2 is that at least one and preferably more, e.g. 1-15, amino acid residues comprising an attachment group for the non-polypeptide moiety ii) has/have been removed, preferably by substitution, from the amino acid sequence. The amino acid residue to be removed is preferably one to which conjugation is disadvantageous, e.g. an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site may result in inactivation or reduced FVII or FVIIa activity of the resulting conjugate due to impaired receptor recognition). In the present context the term "functional site" is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of FVII or FVIIa. Such amino acid residues are a part of the functional site. The functional site may be determined by methods known in the art and is preferably identified by analysis of a structure of the FVIIa complex (See Banner et al., Nature 1996; 380:41-46)

Preferably, the conjugate of the invention has one or more of the following improved properties:

Increased functional *in vivo* half-life, increased plasma half-life, reduced renal clearance and reduced sensitivity to proteolytic degradation as compared to rFVIIa (e.g.NovoSeven®).

Normally, the increased functional *in vivo* half-life is obtained as a consequence of the conjugate having a reduced susceptibility to renal clearance as compared to rFVIIa, NovoSeven®. The reduced susceptibility to renal clearance is obtained as a consequence of the molecular weight, size, shape/rigidity, net charge and other characteristics of the conjugate being changed as compared to the unconjugated polypeptide. Typically, the conjugate according to the invention has a molecular weight of at least 67 kDa, preferably at least 70 kDa, although also a lower molecular weight may give rise to a reduced renal clearance.

In a further embodiment the non-polypeptide moiety of the conjugate of the invention is a molecule which has lysine as an attachment group, wherein the amino acid sequence of the polypeptide i) differs from the amino acid sequence shown in SEQ ID NO 2 in removal, preferably by substitution, of at least one amino acid residue occupying a position selected from the group consisting of K18, K32, K38, K62, K85, K109, K137, K143, K148, K157, K161, K197, K199, K316, K337, K341 and K389, and more preferably at least one amino acid residue occupying a position selected from the group consisting of K18, K62, K85, K85, K197 and K389. The lysine residue may be substituted for any other amino acid residue, but is preferably substituted for R,Q,N or H, more preferably R. In addition, one or more non-naturally occurring glycosylation sites may be introduced as described hereinafter.

In an alternative embodiment of the present invention, the non-polypeptide moiety is attached to a cysteine residue. The cysteine residue is one which has been introduced into the sequence, preferably by substitution.

While the non-polypeptide moiety ii) of the conjugate according to this aspect of the invention may be any molecule which, when using the given conjugation method has lysine or a cysteine as an attachment group (such as a carbohydrate moiety, a lipophilic group or an organic derivatizing agent), it is preferred that the non-polypeptide moiety is a polymer molecule. The polymer molecule may be any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule", but is preferably selected from the group consisting of linear or branched polyethylene glycol or polyalkylene oxide. Most preferably, the polymer molecule is mPEG-SPA or mPEG-SC described in WO 90/13540.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, prefererably substitution specified in the other sections herein disclosing specific amino acid modifications.

Conjugate of the invention wherein the non-polypeptide moiety is a carbohydrate moiety

In a further embodiment the non-polypeptide moiety ii) of the conjugate of the invention is a carbohydrate moiety and the polypeptide i) comprises an amino acid sequence that differs from the amino acid sequence shown in SEQ ID NO 2 in that a glycosylation site has been introduced or removed by way of introduction or removal of amino acid residue(s) constituting a part of a glycosylation site.

It will be understood that in order to prepare a conjugate according to this embodiment the polypeptide i) must be expressed in a glycosylating host cell capable of attaching carbohydrate moieties at the glycosylation site(s). Examples of glycosylating host cells are given in the section further below entitled "Coupling to a carbohydrate moiety".

Preferably, the N-residue of the introduced glycosylation site is located in a position, which in the polypeptide with SEQ ID NO 2 is occupied by an amino acid residue having more than 25%, such as more than 50% or even more than 75% of its side chain exposed at the surface of the molecule.

More preferably, or alternatively, the glycosylation site is introduced in a position occupied by a lysine residue, in particular so that the N-residue of the glycosylation site substitutes the lysine residue.

Preferably, an N- glycosylation site has been introduced into the amino acid sequence of the polypeptide i) by way of substitution by one or more amino acid residue. For instance one embodiment of the invention is an active conjugate, wherein the amino acid sequence of the polypeptide i) shown in SEQ ID NO 2 differs in that at least one mutation selected from the group consisting of F4S, F4T, A175S, A175T, I186S, I186T, R202S, R202T, I205S, I205T, P303S, P303T, P10N, Q21N, W41N, G58N, K109N, G117N, G124N, S147T, T267N, L280N, T324S, D334N, K337N, D104N, T106N, V253N, E265N, E270N, G291N and R353N.

More preferably, the amino acid sequence of the polypeptide i) shown in SEQ ID NO 2 differs in at least one mutation selected from the group consisting of F4S, F4T, A175S, A175T, I186S, I186T, R202S, R202T, I205S, I205T, P303S, P303T, P10N, Q21N, W41N, G58N, K109N, G117N, G124N, T267N, L280N, D334N, K337N, D104N, T106N, V253N, E265N, E270N, G291N and R353N.

In addition to a carbohydrate molecule, the conjugate according to the aspect of the invention described in the present section may contain additional non-polypeptide moieties, in particular a polymer molecule, as described in the present application, conjugated to one or more attachment groups present in the polypeptide part of the conjugate.

It will be understood that any of the amino acid changes, in particular substitutions, specified in this section can be combined with any of the amino acid changes, in particular substitutions, specified in the other sections herein disclosing specific amino acid changes.

An inactive conjugate

The active conjugate described in the preceeding sections may be rendered inactive by removing at least one amino acid residue, shown in SEQ ID NO 2, occupying a position selected from the group consisting of R152, I153, S344, D242 and H193, by complexing to a serine proteinase inhibitor, by carbamylation of the α amino acid group I153, and/or introducing at least one glycosylation site in a position selected so that the subsequent glycosylation inactivated the conjugate.

The serine inhibitor protein may be an organophosphor coumpound, a sulfanylfluoride, apeptide halomethylketone, preferably a Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-Glu-Glu-Arg chlormethylketone, Dansyl-Phe-Phe-Arg chlormethylketone or a Phe-Phe-Arg chlormethylketone.

Non-polypeptide moiety of the conjugate of the invention

As indicated further above the non-polypeptide moiety of the conjugate of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, a carbohydrate moiety (by way of *in vivo* glycosylation) and an organic derivatizing agent. All of these agents may confer desirable properties to the polypeptide part of the conjugate, in

particular an increased functional *in vivo* half-life and/or an increased plasma half-life. The polypeptide part of the conjugate is normally conjugated to only one type of non-polypeptide moiety, but may also be conjugated to two or more different types of non-polypeptide moieties, e.g. to a polymer molecule and a carbohydrate moiety, to a lipophilic group and a carbohydrate moiety, to an organic derivatizing agent and a carbohydrate moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties may be done simultaneous or sequentially.

Methods of preparing a conjugate of the invention

In the following sections "Conjugation to a lipophilic compound", "Conjugation to a polymer molecule", "Conjugation to a carbohydrate moiety" and "Conjugation to an organic derivatizing agent" conjugation to specific types of non-polypeptide moieties is described.

Conjugation to a lipophilic compound

The polypeptide and the lipophilic compound may be conjugated to each other, either directly or by use of a linker. The lipophilic compound may be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamine, a carotenoide or steroide, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl-, aryl-, alkenyl- or other multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker may be done according to methods known in the art, e.g. as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

Conjugation to a polymer molecule

The polymer molecule to be coupled to the polypeptide may be any suitable polymer molecule, such as a natural or synthetic homo-polymer or heteropolymer, typically with a molecular weight in the range of 300-20,000 Da, more preferably in the range of 300-10,000 Da, most preferably in the range of 300-5000 Da.

Examples of homo-polymers include a polyol (i.e. poly-OH), a polyamine (i.e. poly-NH_2) and a polycarboxylic acid (i.e. poly-COOH). A hetero-polymer is a polymer, which comprises one or more different coupling groups, such as, e.g., a hydroxyl group and an amine group.

Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG), methoxypolyethylene glycol (mPEG) and polypropylene glycol (PPG), PEG-glycidyl ether (Epox-PEG), PEG-oxycarbonyl imidazole (CDI-PEG), branced PEGs, polyvinyl alcohol (PVA), polycarboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-malic acid anhydride, dextran including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increase functional *in vivo* half-life and/or plasma half-life. In addition, human albumin or other abundant plasma proteins are considered useful for increasing the functional *in vivo* half-life or plasma half-life of FVII, when conjugated to FVII.

The unique property of polyalkylene glycol-derived polymers of value for modification of polypeptides for therapeutic applications is general biocompatibility. The polymers have various water solubility properties and are non-toxic. They are non-antigenic, non-immunogenic, have long circulation half-life in the blood and are easily excreted from living organisms.

Preferably, PEG, and especially mPEG is used since such polymer molecules have only few reactive groups capable of cross-linking compared, e.g., to polysaccharides such as dextran, and the like. In particular, a non-toxic polymer molecule such as (m)polyethylene glycol ((m)PEG) is of interest since the chemistry for its covalent coupling to attachment group(s) of the FVII is relatively simple because it has only one reactive end capable of conjugating with the polypeptide. Consequently, the risk of cross-linking is eliminated and the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to

control. Activated mPEG, with a functional group reactive towards amines on a protein molecule, is used in most cases.

To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e. with reactive functional groups. Normally, the polymer molecules are purchased in activated form, e.g. from Shearwater Polymers, Huntsville, AL, USA. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g. as disclosed in WO 90/13540.

The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g. as described in the following references (which also describe suitable methods for activation of polymer molecules): R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G.T. Hermanson *et al.*, (1993), "Immobilized Affinity Ligand Techniques", Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the FVII as well as the functional groups of the polymer (e.g. being amino, hydroxyl, carboxyl, aldehyde or sulfydryl). For instance, PEG vinylsulfone (VS-PEG) is a derivative for selective coupling to sulfhydryl groups.

Furthermore, the PEGylation may be designed so as to introduce an optimal number of polymer molecules. For instance, the proteolytic stability is increased with the number of polymer molecules, whereas the molecular weight of the conjugate is important for reducing renal clearance.

The type and/or number of the non-polypeptide moiety is preferably selected so that the resulting polypeptide conjugate has a reduced renal clearance (for instance as a consequence of the molecular weight being larger than the glomerular filtration cut off value, which is approximately 67 kDa, and/or the net charge, size or shape of the conjugate is one, which results in a reduced renal clearance).

Normally, the polymer conjugation is performed under conditions aiming at reacting all available polymer attachment groups with polymer molecules. Optimal conditions is when the polymer is in excess to obtain a conjugate wherein all available polymer attachment groups react with polymer molecules. Typically, the molar ratio of activated polymer molecules to polypeptide is 1000-1, such as 500-1 or 200-1, preferably 100-1, such as 50-1 or 25-1 in order to obtain optimal reaction. Furthermore, the polymer modification, such as PEGylation, in case of mPEG-SPA, is conveniently carried out at a pH in the range of 7-10, such as in the range of 8-10, in particular in the range of 8-9.

It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski *et al.*, (1977), J. Biol. Chem., 252, 3578-3581; US 4,179,337; Shafer *et al.*, (1986), J. Polym. Sci. Polym. Chem. Ed., 24, 375-378.

Subsequent to the conjugation residual activated polymer molecules are blocked according to methods known in the art, e.g. by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules removed by a suitable method.

Covalent in vitro coupling of glycosides (such as dextran) to amino acid residues of the polypeptide may also be used, e.g. as described, for example in WO 87/05330 and in Aplin etl al., CRC Crit Rev. Biochem., pp. 259-306, 1981.

The *in vitro* coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine-groups to protein- and peptide-bound Gln-residues in a so-called cross-linking reaction. The donor-amine groups can be protein- or peptide-bound e.g. as the ε-amino-group in Lys-residues or it can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., Biochemistry 1996; 35:13072-13080).

TGases, in general, are highly specific enzymes, and not every Gln-residues exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary only few Gln-residues are naturally functioning as TGase substrates but the exact parameters governing which Gln-residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g. substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

Coupling to a carbohydrate moiety

In order to achieve in vivo glycosylation of a FVII molecule comprising one or more glycosylation sites the nucleotide sequence encoding the polypeptide must be inserted in a glycosylating, eucaryotic expression host. The expression host cell may be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment the host cell is a mammalian cell, such as a CHO cell, BHK or HEK, e.g. HEK 293, cell, or an insect cell, such as an SF9 cell, or a yeast cell, e.g. *S. cerevisiae* or *Pichia pastoris*, or any of the host cells mentioned hereinafter.

Coupling to an organic derivatizing agent

Covalent modification of the polypeptide exhibiting FVII activity may be performed by reacting (an) attachment group(s) of the polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonateat pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedionc and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group.

Furthermore, these reagents may react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Blocking of functional site

It has been reported that excessive polymer conjugation can lead to a loss of activity of the polypeptide to which the polymer is conjugated. This problem can be eliminated, e.g., by removal of attachment groups located at the functional site or by blocking the functional site prior to conjugation so that the functional site is blocked during conjugation. The latter strategy constitutes further embodiments of the invention (the first strategy being exemplified further

above, e.g. by removal of lysine residues which may be located close to the functional site). More specifically, according to the second strategy the conjugation between the polypeptide and the non-polypeptide moiety ii) is conducted under conditions where the functional site of the polypeptide i) is blocked by a helper molecule e.g. tissue factor capable of binding to the functional site of the polypeptide i).

Preferably, the helper molecule is one, which specifically recognizes a functional site of the polypeptide, such as a receptor, in particular tissue factor, either full length or a suitably truncated form of tissue factor or two molecules, one being tissue factor the other one being a peptide or peptide inhibitor binding to and thus protecting the area around the catalytic triad (preferably defined as amino acid residues within 10 Å of any atom in the catalytic triad).

Alternatively, the helper molecule may be an antibody, in particular a monoclonal antibody recognizing the FVII polypeptide. In particular, the helper molecule may be a neutralizing monoclonal antibody.

The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety such, as a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide can be recovered with at least a partially preserved functional site.

The subsequent conjugation of the polypeptide having a blocked functional site to a polymer, a lipophilic compound, a sugar moiety, an organic derivatizing agent or any other compound is conducted in the normal way, e.g. as described in the sections above entitled "Conjugation to ....".

Irrespectively of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free from or comprises only a few attachment groups for the non-polypeptide moiety of choice in part(s) of the molecule, where the conjugation to such groups will hamper the desorption of the conjugated polypeptide from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule such as PEG, which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free from conjugatable epsilon amino groups, preferably free from any epsilon amino groups. Accordingly, in a preferred embodiment the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free from any conjugatable attachment groups for the non-polypeptide moiety of choice.

In a further embodiment the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g. reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g. as described in the sections above entitled "Conjugation to ....". This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide conjugate is eluated by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule may be derivatised with a second molecule (e.g. biotin) that can be recognized by a specific binder (e.g. streptavidin). The specific binder may be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate may be released from the helper molecule in any appropriate fashion. Deprotection may be achieved by providing conditions in which the helper molecule dissociates from the functional site of the FVII to which it is bound. For instance, a

complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH. Even more preferred is the use of a conformation specific antibody that recognizes a Ca^{2+} specific conformation of FVII and consequently can be eluted with EDTA under mild conditions.

Attachment of a serine proteinase inhibitor

Attachment of a serine proteinase inhibitor can be performed in accordance to the method mentioned in WO 96/12800.

Methods of preparing a polypeptide of the invention or the polypeptide i) of the conjugate of the invention

The polypeptide of the present invention or the polypeptide part of a conjugate of the invention, optionally in glycosylated form, may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host, e.g. as described in the prior art. Preferably, the host cell is a gammacarboxylating host cell such as a mammalian cell. However, polypeptides of the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

The nucleotide sequence of the invention (SEQ ID NO 1) encoding a polypeptide or the polypeptide part i) of a conjugate of the invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FVII, such as hFVII with the amino acid sequence shown in SEQ ID NO 2 and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or deletion (i.e. removal or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, PNAS 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FVII in the desired transformed host cell.

It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding a polypeptide described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or

culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

The recombinant vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector, in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, CA, USA) and pCI-neo (Stratagene, La Jola, CA, USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (US 4,931,373), the pJSO37 vector described in Okkels, Ann. New York Acad. Sci. 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate *et al.*, "Isolation of the Bovine and Human Genes for Mullerian Inhibiting Substance And Expression of the Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g. , NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., US 5,122,464 and EP 338,841).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include *ura3 and leu2*. For filamentous fungi, selectable markers include *amdS*, *pyrG*, *arcB*, *niaD* and *sC*.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

A wide variety of expression control sequences may be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to

control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g. the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the *Drosophila* minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20;196(4):947-50).

In order to improve expression in mammalian cells a synthetic intron may be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the *lac* system, the *trp* system, the *TAC* or *TRC* system, and the major promoter regions of phage lambda.

The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. US 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy *et al.*, Protein Expression and Purification 4, 349-357 (1993) or human pancreatic lipase (hpl) (Methods in Enzymology 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFVII or the murine Ig kappa light chain signal peptide (Coloma, M (1992) J. Imm. Methods 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. US 4,870,008), a modified carboxypeptidase signal peptide (cf. L.A. Valls *et al.*, Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani *et al.*, Yeast 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide *ompA* (EP581821).

The nucleotide sequence of the invention encoding a FVII polypeptide, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, may or may not also include a nucleotide sequence that encode a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The

signal peptide may be homologous (e.g. be that normally associated with hFVII) or heterologous (i.e. originating from another source than hFVII) to the polypeptide or may be homologous or heterologous to the host cell, i.e. be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide may be prokaryotic, e.g. derived from a bacterium such as *E. coli*, or eukaryotic, e.g. derived from a mammalian, or insect or yeast cell.

Any suitable host may be used to produce the polypeptide or polypeptide part of the conjugate of the invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include grampositive bacteria such as strains of *Bacillus*, e.g. *B. brevis* or *B. subtilis*, *Pseudomonas* or *Streptomyces*, or gramnegative bacteria, such as strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of *Aspergillus*, e.g. *A. oryzae*, *A. niger*, or *A. nidulans*, *Fusarium* or *Trichoderma*. Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and US 5,679,543. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of *Saccharomyces*, e.g. *S. cerevisiae*, *Schizosaccharomyces*, *Klyveromyces*, *Pichia*, such as *P. pastoris* or *P. methanolica*, *Hansenula*, such as *H. Polymorpha* or *Yarrowia*. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, *Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, CA, USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a *Lepidoptora* cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (US 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein may be performed as described by Invitrogen. Examples of suitable mammalian host cells include Chinese hamster ovary (CHO) cell lines, (e.g. CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g. COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g. NS/O), Baby Hamster Kidney (BHK) cell lines (e.g. ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g. HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g. described by Ausbel *et al.* (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, New York, USA. The cultivation of mammalian cells are conducted according to established methods, e.g. as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, New Jersey, USA and Harrison MA and Rae IF, General Techniques of Cell Culture, Cambridge University Press 1997).

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation

(including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

The polypeptides may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989). Specific methods for purifying polypeptides exhibiting FVII activity are described by D. Metcalf and N. A. Nicola in *The hemopoietic colony-stimulating factors*, p. 50-51, Cambridge University Press (1995), by C. S. Bae *et al.*, Appl. Microbiol. Biotechnol, 52:338-344 (1999) and in US4810643.

In a preferred embodiment the polypeptide is purified as a single chain FVII which further is PEGylated. The PEGylated FVII single chain polypeptide is activated by either use of an immobilized enzyme (e.g. factors IIa, IXa, Xa and XIIa) or by autoactivation using a positively charged ion exchange matrix or the like.

Homogeneous preparation of a conjugate of the invention

In a further aspect the invention relates to a substantially homogeneous preparation of a conjugate of the invention. In the present context a "substantially homogeneous preparation" is a preparation containing more than 50%, such as more than 75% and preferably more than 85%, or more than 90% identical conjugates, i.e. having the same degree and nature of conjugation. The substantially homogeneous preparation is conveniently obtained by ensuring that the polypeptide part of the conjugate contains the necessary number of attachment groups, located at the surface of the molecule in such a way that all attachment groups can be conjugated to the non-polypeptide moiety of choice, when the conjugation is performed in the presence of a molar excess of the non-polypeptide moiety relative to the polypeptide. Preferably, the non-polypeptide moiety to be used in this aspect of the invention is a polymer molecule.

Pharmaceutical composition of the invention and its use

In one aspect the active FVIIa polypeptide, conjugate or pharmaceutical composition according to the invention is used for the manufacture of a medicament for treatment of diseases, in particular treatment of patients having diseases resulting in inadequate blood coagulation in response to damage to blood vessels. This group of patients include, but are not limited to, hemophiliacs, hemophiliacs with inhibiotors to FVIII and FIX, patients with thrombocytopenia, patients with liver diseases, or otherwise healthy people with severe bleeding problems, e.g. due to trauma or major surgery, who have developed inhibitors to FVIIa, bleeding disorders such as hemophiliacs and other typically associated with severe tissue damages.

In another aspect the active FVII or FVIIa polypeptide, conjugate or pharmaceutical composition according to the invention is used in a method of treating a mammal having such diseases comprising administering to a mammal in need thereof such a polypeptide, conjugate or pharmaceutical composition.

In another aspect the inactive FVII or FVIIa polypeptide, conjugate or pharmaceutical composition according to the invention is used for the manufacture of a medicament for

treatment of diseases, in particular treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction, thrombotic stroke etc.

In another aspect the inactive FVII or FVIIa polypeptide, conjugate or pharmaceutical composition according to the invention is used in a method of treating a mammal having such diseases, thrombotic stroke etc., comprising administering to a mammal in need thereof such a polypeptide, conjugate or pharmaceutical composition.

The FVII or FVIIa polypeptide of the invention or the conjugate of the invention is administered at a dose approximately paralleling that employed in therapy with rFVII such as NovoSeven®, or at higher dosis. The exact dose to be administered depends on the circumstances. Normally, the dose should be capable of preventing or lessening the severity or spread of the condition or indication being treated. It will be apparent to those of skill in the art that an effective amount of a polypeptide, conjugate or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide or conjugate or composition is administered alone or in conjunction with other therapeutic agents, the plasma half-life of the compositions, and the general health of the patient. Preferably, the polypeptide, conjugate, preparation or composition of the invention is administered in an effective dose, in particular a dose which is sufficient to normalise the coagulation disorder.

The polypeptide or conjugate of the invention is preferably administered in a composition including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is ~~administered. Such pharmaceutically acceptable carriers and excipients are well-known in the~~ art.

The polypeptide or conjugate of the invention can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described by Remington's Pharmaceutical Sciences by E.W.Martin (Mark Publ. Co., 16th Ed., 1980).

The pharmaceutical composition of the polypeptide or conjugate of the invention may be formulated in a variety of forms, including a stable soluble liquid, gel, lyophilised, or any other suitable form. Preferably, the pharmaceutical composition of the polypeptide or the conjugate of the invention is lyophilised or in a stable soluble form. The polypeptide or the conjugate may be lyophilised by a variety of procedures known in the art. A polypeptide or the conjugate may be a stable soluble form by the removal of proteolytic degradation sites. The advantage of obtaining a stable soluble preparation lies in easier handling for the patient and in the case of emergencies quicker action, which potentially can become life saving. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

The pharmaceutical composition containing the polypeptide or conjugate of the invention may be administered orally, intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, by inhalation, or in any other acceptable manner, e.g. using PowderJect or ProLease technology. The preferred mode of administration is intravenously.

The pharmaceutical composition of the invention may be administered in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical composition or may be administered separately from the polypeptide or conjugate of the invention, either concurrently or in accordance with any other acceptable treatment schedule. In addition, the polypeptide, conjugate or pharmaceutical composition of the invention may be used as an adjunct to other therapies.

The invention is further described in the following examples. The examples should not, in any manner, be understood as limiting the generality of the present specification and claims.

METHODS

Methods used to determine the amino acids to be modified

Accessible Surface Area (ASA)

The computer program Access (B. Lee and F.M.Richards, J. Mol.Biol. 55: 379-400 (1971)) version 2 (Copyright (c) 1983 Yale University) are used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the centre of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

Fractional ASA of side chain

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide. See Hubbard, Campbell & Thornton (1991) J.Mol.Biol.220,507-530. For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table are used as standard 100% ASA for the side chain:

Ala	69.23	$Å^2$
Arg	200.35	$Å^2$
Asn	106.25	$Å^2$
Asp	102.06	$Å^2$
Cys	96.69	$Å^2$
Gln	140.58	$Å^2$
Glu	134.61	$Å^2$
Gly	32.28	$Å^2$
His	147.00	$Å^2$
Ile	137.91	$Å^2$
Leu	140.76	$Å^2$
Lys	162.50	$Å^2$
Met	156.08	$Å^2$
Phe	163.90	$Å^2$
Pro	119.65	$Å^2$
Ser	78.16	$Å^2$
Thr	101.67	$Å^2$
Trp	210.89	$Å^2$
Tyr	176.61	$Å^2$
Val	114.14	$Å^2$

Residues not detected in the structure are defined as having 100% exposure as they are thought to reside in flexible regions. The gamma-carboxy glutamic acids at positions 6,7,14,16,19,20,25,26,29 and 35 are all defined as being 100% exposed.

Determining distances between atoms

The distance between atoms is most easily determined using molecular graphics software e.g. InsightII v. 98.0, MSI INC.

Catalytic site region

The catalytic site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344).

Determination of tissue factor binding site

The receptor-binding site is defined as comprising of all residues having their accessible surface area changed upon receptor binding. This is determined by at least two ASA calculations; one on the isolated ligand(s) in the ligand(s)/receptor(s) complex and one on the complete ligand(s)/receptor(s) complex.

Methods for testing FVII and FVIIa properties

Measurement of the in vivo half-life of conjugated and unconjugated rFVII and variants thereof

Measurement of *in vivo* biological half-life can be carried out in a number of ways as described in the literature. An example of an assay for the measurement of *in vivo* half-life of rFVIIa or variants thereof is described in FDA reference number 96-0597. Briefly, FVII clotting activities are measured in plasma drawn prior to and during a 24-hour period after administration of the conjugate, polypeptide or composition. The median apparent volume of distribution at steady state is measured and the median clearance determined.

Measurement of the reduced sensitivity to proteolytic degradation

Proteolytic degradation can be measured using the assay described in US 5,580,560, Example 5, where proteolysis is either autoproteolysis or the result of addition of purified Cathepsin G.

Furthermore, reduced proteolysis can be tested in an in vivo model using radiolabelled samples and comparing proteolysis of wild type and conjugates by withdrawing blood samples and sujecting these to SDS-PAGE and autoradiography.

Irrespectively of the assay used for determining proteolytic degradation, "reduced proteolytic degradation" is intended to mean a measurable reduction in cleavage compared to that obtained by non-conjugated wild type FVIIa as measured by gel scanning of Coomassie stained SDS-PAGE gels or as measured by conserved catalytic activity in comparison to wild type using the chomogenic assay described above.

Determination of the molecular size of rFVII and variants thereof

The molecular weight of conjugated or unconjugated rFVII or variants thereof is determined by either SDS-PAGE, gel filtration, matrix assisted laser desorption mass spectrometry or equilibrium centrifugation

Methods of purification of single chain FVII

Single chain FVII can be purified and activated to two-chain FVIIa by a number of methods as described in the literature (Broze and Majerus, 1980, J. Biol. Chem. 255:1242-47 and Hedner and Kisiel, 1983, J.Clin.Invest. 71:1836-41). Another method whereby single chain FVII can be purified is by incorporation of Zn ions during purification as described in US 5,700,914.

It is advantageous to first purify FVII in its single chain form, then PEGylate and last activate by one of the methods described above or by autoactivation as described by Pedersen et al, 1989, Biochemistry 28: 9331-36. The advantage of carrying out PEGylation before activation is that PEGylation of the new aminoterminal formed by cleavage of R152-I153 is avoided. PEGylation of this new amino terminal would render the molecule inactive since the formation of a hydrogen bond between D242 and the amino terminal of I153 is necessary for activity.

Methods of measuring the anticoagulant activity

The anticoagulant activity of an inactive FVII or FVIIa conjugate can be measured using the one-stage clotting assay where the inactive conjugate competes with wild type FVII for a limited amount of relipidated tissue factor. The assay is performed essentially as described in WO92/15686, example III, which is hereby incorporated as reference. The ability of the inactive

conjugate to prolong the clotting time of wild type FVII is recorded and taken as a measure of anticoagulant activity.

Method of measuring the catalytic activity

The ability of the conjugates to cleave small peptide substrates can be measured using the chromogenic substrate S-2288 (Ile-Pro-Arg-p-nitroanilide). Recombinant FVIIa is diluted in 0.1 M Tris, 0.1 M NaCl, 5 mM $CaCl_2$, pH 8.3 containing 0.1 % BSA. The reaction is started by addition of substrate to 1 mM and absorption at 405 nm measured after incubation 30 min at 37°C.

Method of measuring the clotting activity

FVIIa activity is measured using a standard one-stage clotting assay essentially as described in WO92/15686. Briefly, the sample to be tested is diluted in 50 mM Tris pH 7.5, 0.1 % BSA and 100 ul is incubated with 100 ul of FVII deficient plasma and 200 ul of thromboplastin C containing 11.8 mM Ca^{++}. Clotting times are measured and compared to a standard curve using a pool of citrated normal human plasma in serial dilution.

EXAMPLES

EXAMPLE 1:

The X-ray structure of hFVIIa in complex with soluble tissue factor by Banner et.al., J Mol Biol, 1996; 285:2089 is used for this example. It is noted that the numbering of residues in the reference does not follow the sequence. Here we have used the sequential numbering according to SEQ ID NO 2. The gamma-carboxy glutamic acids at positions 6,7,14,16,19,20,25,26,29 and 35 are all here named GLU (three letter abbreviation) or E (one letter abbreviation). The residues 143-152 are not present in the structure.

<u>Surface exposure:</u>
Performing fractional ASA calculations on FVII fragments alone combined with the definition of accessibilities of non standard and/or missing residues described in the methods resulted in the following residues having more than 25% of their side chain exposed to the surface: A1, N2, A3, F4, L5, E6, E7, L8, R9, P10, S12, L13, E14, E16, K18, E19, E20, Q21, S23, F24, E25, E26, R28, E29, F31, K32, D33, A34, E35, R36, K38, L39, W41, I42, S43, S45, G47, D48, Q49, A51, S52, S53, Q56, G58, S60, K62, D63, Q64, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, T83, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, E99, S103, D104, H105, T106, G107, T108, K109, S111, R113, E116, G117, S119, L120, L121, A122, D123, G124, V125, S126, T128, P129, T130, V131, E132, I140, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, V158, P160, K161, E163, L171, N173, G174, A175, N184, T185, I186, H193, K197, K199, N200, R202, N203, I205, S214, E215, H216, D217, G218, D219, S222, R224, S232, T233, V235, P236, G237, T238, T239, N240, H249, Q250, P251, V253, T255, D256, E265, R266, T267, E270, R271, F275, V276, R277, F278, L280, L287, L288, D289, R290, G291, A292, T293, L295, E296, N301, M306, T307, Q308, D309, L311, Q312, Q313, R315, K316, V317, G318, D319, S320, P321, N322, T324, E325, Y326, Y332, S333, D334, S336, K337, K341, G342, H351, R353, G354, Q366, G367, T370, V371, G372, R379, E385, Q388, K389, R392, S393, E394, P395, R396, P397, G398, V399, L400, L401, R402, P404, P406. The following residues had more than 50% of their side chain exposed to the surface: A1, A3, F4, L5, E6, E7, L8, R9, P10, E14, E16, K18, E19, E20, Q21, S23, E25, E26, E29, K32, A34, E35, R36, K38, L39, I42, S43, G47, D48, A51, S52, S53, Q56, G58, S60, K62, L65, Q66, S67, I69, F71, L73, P74, A75, E77, G78, R79, E82, H84, K85, D86, D87, Q88, L89, I90, V92, N93, E94, G97, T106, G107, T108, K109, S111, E116, S119, L121, A122, D123, G124, V131, E132, L141, E142, K143, R144, N145, A146, S147, K148, P149, Q150, G151, R152, G155, K157, P160, N173, G174, A175, K197, K199, N200,

R202, S214, E215, H216, G218, R224, V235, P236, G237, T238, H249, Q250, V253, D256, T267, F275, R277, F278, L288, D289, R290, G291, A292, T293, L295, N301, M306, Q308, D309, L311, Q312, Q313, R315, K316, G318, D319, N322, E325, D334, K341, G354, G367, V371, E385, K389, R392, E394, R396, P397, G398, R402, P404, P406.

<u>Tissue factor binding site:</u>
Performing ASA calculations as described in the methods the following residues in human FVII change their ASA in the complex, these were defined as comprising the receptor binding site: L13, K18, F31, E35, R36, L39, F40, I42, S43, S60, K62, D63, Q64, L65, I69, C70, F71, C72, L73, P74, F76, E77, G78, R79, E82, K85, Q88, I90, V92, N93, E94, R271, A274, F275, V276, R277, F278, R304, L305, M306, T307, Q308, D309, Q312, Q313, E325, R379.

<u>Active site region:</u>
The active site region is defined as any residues having at least one atom within 10 Å of any atom in the catalytic triad (residues H193, D242, S344): I153, Q167, V168, L169, L170, L171, Q176, L177, C178, G179, G180, T181, V188, V189, S190, A191, A192, H193, C194, F195, D196, K197, I198, W201, V228, I229, I230, P231, S232, T233, Y234, V235, P236, G237, T238, T239, N240, H241, D242, I243, A244, L245, L246, V281, S282, G283, W284, G285, Q286, T293, T324, E325, Y326, M327, F328, D338, S339, C340, K341, G342, D343, S344, G345, G346, P347, H348, L358, T359, G360, I361, V362, S363, W364, G365, C368, V376, Y377, T378, R379, V380, Q382, Y383, W386, L387, L400, F405.

CLAIMS

1. A conjugate comprising
   i) an FVII or FVIIa polypeptide, wherein the amino acid sequence of the polypeptide differs from the amino acid sequence of wild-type FVII or FVIIa shown in SEQ ID NO 2 in at least one introduced or removed amino acid residue comprising an attachment group for the non-polypeptide moiety of ii), and
   ii) a non-polypeptide moiety.
2. The conjugate according to claims 1, wherein the amino acid sequence of the polypeptide differs in 1-15 amino acid residues from the amino acid sequence as shown in SEQ ID NO 2.
3. The conjugate according to claim 1 or 2, wherein one difference between the amino acid sequence of the polypeptide i) and the amino acid sequence shown in SEQ ID NO 2 is that at least one amino acid residue comprising an attachment group for the non-polypeptide moiety ii) has been introduced into the amino acid sequence.
4. The conjugate according to claim 3, wherein 1-15 amino acid residues comprising an attachment group for the non-polypeptide moiety has/have been introduced into the amino acid sequence.
5. The conjugate according to claim 3 or 4, wherein the non-polypeptide moiety is a molecule which has a lysine residue as an attachment group and wherein the amino acid sequence of the polypeptide shown in SEQ ID NO 2 differs in that at least one amino acid residue ~~selected from the group consisting of K18, K32, K38, K62, K85, K109, K137, K143, K148,~~ K157, K161, K197, K199, K316, K337, K341 and K389 has been substituted.
6. The conjugate according to claim 5, wherein at least one of K18, K62, K85 K62, K85, K197 and K389 is substituted for another amino acid residue.
7. The conjugate according to claim 5 or 6, wherein K is substituted for R, Q, N or H, preferably R.
8. The conjugate according to any of claims 1-7, wherein at least one *in vivo* glycosylation site has been introduced by substitution of an amino acid residue shown in SEQ ID NO 2, the substitution being selected from the group consisting of F4S, F4T, A175S, A175T, I186S, I186T, R202S, R202T, I205S, I205T, P303S, P303T, P10N, Q21N, W41N, G58N, K109N, G117N, G124N, S147T, T267N, L280N, T324S, D334N, K337N, D104N, T106N, V253N, E265N, E270N, G291N and R353N.
9. The conjugate according to claim 8, wherein two or more glycosylation sites have been introduced.
10. The conjugate according to any of claims 1-9, wherein the polypeptide i) is an inactive FVII or FVIIa or FVIIa polypeptide.
11. The conjugate according to claim 10, wherein the amino acid sequence of the polypeptide differs from the amino acid sequence shown in SEQ ID NO 2 in at least one amino acid residue selected from the group consisting of R152, I153, S344, D242 and H193.
12. The conjugate according to claim 10 or 11, wherein the catalytic site of the polypeptide is complexed with a serine protease inhibitor or amino acid residue I 153 is carbamylated.
13. The conjugate according to any of claims 1-12 wherein the non-polypeptide moiety is a polymer molecule.
14. The conjugate according to claim 13, wherein the polymer molecule is selected from a group consisting of linear or branched polyethylene glycol and polyalkylene oxide.
15. The conjugate according to any of claims 1-3, wherein the non-polypeptide moiety is a carbohydrate moiety and wherein the amino acid sequence of the polypeptide i) comprises at least one mutation selected from the group consisting of F4S, F4T, A175S, A175T, I186S, I186T, R202S, R202T, I205S, I205T, P303S, P303T, P10N, Q21N, W41N, G58N, K109N, G117N, G124N, S147T, T267N, L280N, T324S, D334N, K337N, D104N, T106N, V253N, E265N, E270N, G291N and R353N.
16. The conjugate according to claim 15, comprising at least one mutation selected from the group consisting of F4S, F4T, A175S, A175T, I186S, I186T, R202S, R202T, I205S, I205T,

P303S, P303T, P10N, Q21N, W41N, G58N, K109N, G117N, G124N, T267N, L280N, D334N, K337N, D104N, T106N, V253N, E265N, E270N, G291N and R353N is substituted.

17. The conjugate according to claim 15 or 16, wherein the amino acid differs in 1-15 amino acid residues from the amino acid sequence shown in SEQ ID NO 2.
18. The conjugate according to claim 17, wherein two or more glycosylation sites have been introduced.
19. The conjugate according to any of claims 15-18, wherein the conjugate is inactive.
20. The conjugate according to claim 19, wherein the amino acid sequence of the polypeptide differs from the amino acid sequence shown in SEQ ID NO 2 in that at least one amino acid residue selected from the group consisting of R152, I153, S344, D242 and H193 has been substituted for another amino acid residue.
21. The conjugate according to claim 19 or 20, wherein the catalytic site of the polypeptide is complexed with a serine protease inhibitor or amino acid residue I153 is carbamylated.
22. The conjugate according to any of claims 1-11, wherein the non-polypeptide moiety is one, which is capable of increasing the functional *in vivo* half-life of the factor FVII polypeptide and/or reducing sensitivity to proteolytic degradation.
23. The conjugate according to any of claims 1-22, wherein the non-polypeptide moiety is selected from the group consisting of a lipophilic compound, a sugar moiety and an organic derivatizing agent.
24. A substantially homogenous preparation of a conjugate according to claim 1-23.
25. ~~The conjugate according to any of claims 1-24, wherein the conjugate has a molecular weight~~ of at least 67 kDa.
26. The polypeptide part i) of the conjugate according to claims 1-12 and 15-21.
27. A nucleotide sequence encoding a polypeptide according to claim 26.
28. An expression vector harbouring a nucleotide sequence according to claim 27.
29. A host cell comprising a nucleotide sequence according to claim 27 or an expression vector according to claim 28.
30. The host cell according to claim 29, which is a CHO, BHK or a HEK293 cell.
31. A method of increasing the functional *in vivo* half-life and/ or plasma half-life and/or a reducing proteolysis of a factor FVII polypeptide, which method comprises introducing, preferably by substitution, an amino acid residue as defined in claims 1-23 and subjecting the resulting modified polypeptide to conjugation with the non-polypeptide moiety.
32. The method according to claim 31, wherein the non-polypeptide moiety is selected from the group consisting of a polymer molecule, a carbohydrate moiety, a lipophilic group and an organic derivatizing agent.
33. A method for preparing a conjugate according to any of claims 13-23, wherein the polypeptide part of the conjugate is allowed to react with the molecule to which it is to be conjugated under conditions conducive for the conjugation to take place, and the conjugate is recovered.
34. The method according to claim 33, wherein the polypeptide part is a purified single amino acid chain, the non-polypeptide part is attached after purification, and the purified conjugate then undergoes activation.
35. A pharmaceutical composition comprising a) a polypeptide according to claim 26 or a conjugate according claims 1-23 and b) a pharmaceutically acceptable diluent, carrier or adjuvant.
36. A pharmaceutical composition according to 35 which is lyophilised.
37. A pharmaceutical composition according to 35 which is a stable liquid formulation.
38. An inactive polypeptide according to claim 26, a polypeptide conjugate according to claims 1-23 or a pharmaceutical composition according to claims 35-37 for the treatment of diseases, in particular treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction, thrombotic stroke.
39. Use of an inactive polypeptide according to claim 26, a conjugate according to any of claims 10-14 or 19-25 or a pharmaceutical composition according to claims 35-37 for the

manufacture of a medicament for treatment of diseases, in particular treatment of patients being in hypercoagulable states, such as patients with sepsis, in risk of myocardial infarction, thrombotic stroke.

40. An active polypeptide according to any of claim 26, a polypeptide conjugate according to claims 1-9, 13-18, or 22-25 or a pharmaceutical composition according to claims 35-37 for the treatment of diseases, in particular diseases resulting in inadequate blood coagulation in response to damage to blood vessels including hemophiliacs, hemophiliacs with inhibitors to FVIII and FIX, thrombocytopenia, liver diseases, or otherwise healthy people with severe bleeding problems, e.g. due to trauma or major surgery.

41. Use of an active polypeptide according to claim 26, a conjugate according to any of claims 1-9, 13-18, or 22-25, for the manufacture of a medicament for treatment of diseases, in particular diseases resulting in inadequate blood coagulation in response to damage to blood vessels including hemophiliacs, hemophiliacs with inhibitors to FVIII and FIX, thrombocytopenia, liver diseases, or otherwise healthy people with severe bleeding problems, e.g. due to trauma or major surgery.

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